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# "In Vivo Role of Pseudomonas aeruginosa Toxins and Host Response"

by

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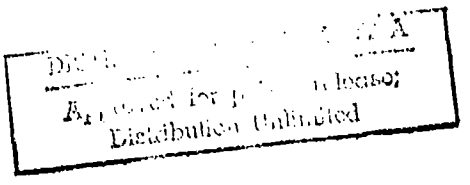


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13. ABSTRACT

Five standard methods of extracting endotoxin were employed in an effort to establish its presence in Pseudomonas aeruginosa as well as to make comparative evaluations of its biological and chemical properties. Of the five preparations, aqueous phenol extracted endotoxin exhibited the greatest degree of lethality. The LD50 was 450 µg dry weight when administered intravenously and 840 µg intraperitoneally. No lethality was observed when endotoxin was administered intranasally. Lethality appeared to be associated with the core region of the lipopolysaccharide molecule, while no correlation between lethality and lipid content was detected.

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## ABSTRACT

Attempts to obtain toxic preparations of endotoxin from *Pseudomonas aeruginosa* ($C_9$) were initiated by employing five different extraction procedures which were subsequently assayed for mouse lethality using a variety of routes. The standard methods of extraction were the aqueous phenol, trichloroacetic acid, ethylenediamine-tetraacetate-lysozyme, ethyl ether and hot water procedures. The aqueous phenol preparation was found to be the most toxic and exhibited an $LD_{50}$ value of 450 µg dry weight when administered intravenously and 840 µg intraperitoneally. No lethality was observed when endotoxin was administered intranasally. The second most lethal preparation was obtained by the trichloroacetic acid extraction and yielded $LD_{50}$ values of 589 µg intravenously and 947 µg intraperitoneally. The other three preparations were considerably less lethal. Comparative quantitative determinations of chemical constituents known to be associated with bacterial endotoxins was carried out on each extraction product with considerable variation in chemical content noted. The aqueous phenol and trichloroacetic acid preparation tended to have a higher content of those carbohydrates associated with the core region of the lipopolysaccharide molecule than did the lesser toxic preparations. No correlation between lethality and lipid content as determined by the alkaline hydroxylamine procedure was observed.

# INTRODUCTION

Over the years, an extensive literature has accumulated regarding the detection and characterization of endotoxin from many gram negative bacteria. However, studies of this nature have been relatively rare or unsuccessful with *Pseudomonas aeruginosa*. Consequently, some investigators felt that little or no endotoxin was present in this organism, and if present, was relatively non-toxic when administered to experimental animals (12, 21, 25). More recent studies are somewhat contradictory in nature, but do suggest that extracted lipopolysaccharides from *P. aeruginosa* may exhibit lethality in mice (19, 24, 25). Lately, *P. aeruginosa* has become a more serious clinical problem and has begun to be one of the more prevalent organisms encountered in nosocomial infections. In addition, the mechanism by which this organism produces its toxic effects on the host has not been firmly established, although a number of extracellular substances have been investigated as possible virulence factors (21, 22, 23). Since the *in vivo* processes seem quite complex, it seemed essential that the role of endotoxin in these infectious processes be elucidated. Consequently, a comprehensive inquiry into the status of endotoxin in *P. aeruginosa* was undertaken in an effort to establish both its presence in the cells as well as its subsequent characterization. Five different, but standard extraction procedures were employed so that valid comparisons could be made regarding the effectiveness of each procedure in yielding toxic preparations. The endotoxin from *P. aeruginosa* Cg was extracted by the following methods: aqueous phenol (38), trichloroacetic acid (3),

ethylenediaminetetraacetate-lysozyme (19), ethyl ether (15), and hot water (30). The $LD_{50}$ value of each extraction product was determined using female white mice with three routes of administration. In addition, a chemical characterization of the various preparations were performed in an effort to determine whether there was some degree of correlation between their chemical composition and degree of lethality.

## MATERIALS AND METHODS

*Culture.* A strain of *P. aeruginosa* Cg isolated from a patient at Children's Hospital, Detroit, Michigan, was employed in this study. Stock cultures of the organism were maintained on tryptose agar slants (Difco).

*Cultivation.* Cells used in these studies were grown in 15 liters of media of the following composition per liter: sodium glutamate, 20 g; glucose, 5 g; $Na_2HPO_4$, 5.6 g; $KH_2PO_4$, 0.25 g; $MgSO_4.7\ H_2O$, 0.1 g; $Ca(NO_3)_2$ 10 mg; $FeSO_4.7\ H_2O$, 50 µg. The sodium glutamate was filter-sterilized and added to the remaining components which had been autoclaved at 15 lb for 30 min. The final pH of the media was 7.6. A 500 ml starter culture was grown in the same media for 18 hr at 37 C. This culture was then added to the fermenter jar which had been equilibrated to 35 C in a New Brunswick fermenter, Model FS-314. The culture was aerated at a rate of 2 liters of air per min and agitated by a paddle stirring at 300 rpm. Cells were harvested by centrifugation after 18 hr incubation. The cells were washed three times in distilled water and frozen until needed.

*Extraction Procedures.* The five standard extraction procedures employed were the aqueous phenol (38), trichloroacetic acid (3), ethylenediaminetetraacetate-lysozyme (19), ethyl ether (15), and hot water (30) methods. The endotoxin obtained by these various extraction procedures were dialyzed against distilled water, precipitated in the cold with ethanol, and lyophilized.

*Animal Studies.* The lyophilized material from each extraction procedure was suspended in sterile pyrogen-free saline. The $LD_{50}$ values of the individual endotoxins was determined using Carworth CF-1 female white mice weighing between 18-25 gm. Three routes of administration were used with five animals per dilution. The animals received 1 ml intraperitoneally, 0.5 ml intravenously, and 0.05 ml intranasally. The control animals received saline. Animals receiving material intranasally were first lightly anesthesized with a mixture of alcohol, chloroform, and ether (1:2:3). Following administration of endotoxin, the animals were observed at various time intervals up to 72 hr. The $LD_{50}$ values were calculated by the method of Reed and Muench (29) from the total number of deaths occurring at 48 hr. Gross pathology as well as microscopic examination of tissues taken from all major organs were also evaluated.

*Acid Hydrolysis.* The acid hydrolysis of samples of endotoxin was carried out in ampules sealed after degassing. The samples were heated in a temp block module heater (Lab-Line Instruments) and mineral oil was added to the wells of the block to insure even distribution of heat. The samples were then heated at temperatures and for lengths of time as prescribed by the various analytical methods.

*Total carbohydrates.* Prior to evaluation of total carbohydrates, samples were hydrolyzed in 6 N HCl for 20 hr at 100 C. Samples of hydrolyzed material were assayed for total carbohydrate by the anthrone (10), cysteine-$H_2SO_4$ (8), and phenol-$H_2SO_4$ (11) methods. Glucose was used as the standard and the values were corrected for loss due to

hydrolysis.

*Pentoses.* Pentoses were determined by the cysteine-$H_2SO_4$ procedure using xylose and ribose as standards (7).

*Methylpentoses.* Rhamnose was the standard for determination of methyl pentoses as determined by the cysteine-$H_2SO_4$ method as modified by Dische (6). Samples of endotoxin were hydrolyzed for 7 hr with 2 N HCl.

*Heptose.* Heptose was determined on samples which were hydrolyzed in 1 N HCl for 2 hr by the cysteine-$H_2SO_4$ procedure as modified by Osborn (28). The standard used was sedoheptulose.

*2-Keto-3-deoxy-octanoate (KDO).* Ketodeoxy sugars were determined by the thiobarbituric acid procedure of Weissbach and Hurwitz (37) as modified by Osborn (28). Samples for analysis were hydrolyzed in 0.02 N $H_2SO_4$ for 20 min at 100 C. The standard used was 2-deoxyribose.

*Hexosamine.* Samples of endotoxin were hydrolyzed for 2 hr at 100 C in 2 N HCl. The hydrolysates were evaporated to dryness, dissolved in 3 ml of acidified distilled water, and passed through a 25 cm x 10 mm Dowex 50 column in the $H^+$ form (2). After washing the column with 10 ml of distilled water the hexosamines were eluted with 2 N HCl. The hexosamine content of the eluted material was determined by the procedure of Rondle and Morgan with glucosamine and galactosamine standards (32).

*Protein and Micronitrogen.* The protein content in the various endotoxin preparations was estimated by the Lowry procedure as modified

by Zak and Cohen (39). Bovine serum albumin served as the standard. The amount of protein was also estimated from values obtained by micro-nitrogen determination (35).

*Lipid.* The comparative quantities of lipid was estimated on the basis of fatty acid content. Fatty acids were determined by the alkaline hydroxylamine procedure as described by Haskins (18). Tripalmitin was used as the standard.

*Phosphate.* Total phosphate was determined after hydrolysis by the method of Fiske and Subbarow (14), and inorganic phosphate by the method of Baginski *et al* (1).

*Nucleic acids.* The quantity of deoxynucleic acid (DNA) present in the five endotoxin preparations was determined by the dephenylamine reaction (4). The standard curve was prepared using highly purified salmon sperm DNA. The amount of ribonucleic acid (RNA) was evaluated by the orcinol procedure with purified RNA as standard (33). The values for RNA as determined by this procedure were corrected for interference due to DNA.

## RESULTS

*Animal Studies.* A comprehensive *in vivo* and *in vitro* characterization of the endotoxin extracted from *P. aeruginosa* by five different extraction procedures was initiated. Initial studies were centered upon possible lethality in mice. Of the five preparations, the phenol-water and the TCA preparations were found to be the most lethal to the animals. The phenol-water product had an $LD_{50}$ value of 840 µg dry weight when injected intraperitoneally (i.p.) and 450 µg intravenously (i.v.). However, a concentration of 1 mg in a volume of 0.05 ml did not kill the animals when administered intranasally. Concentrations above this amount could not be effectively solubilized. The TCA preparation represented the next most toxic product. An $LD_{50}$ of 947 µg i.p. and 589 i.v. was obtained with this preparation. The ethyl ether extracted endotoxin was found to have an intermediate position with regard to lethality. When given by the i.p. route, 1755 µg were required to kill 50% of the animals; on the other hand, 1652 µg was the $LD_{50}$ by the i.v. route. The extraction products obtained by EDTA-lysozyme and hot water were regarded as relatively non-lethal since very high concentrations were required to elicit mouse lethality. The $LD_{50}$ values obtained from the five extraction products are summarized in Table 1.

In animals injected both i.v. or i.p. with material from the phenol-water and TCA extraction procedures, shock symptoms generally appeared within 6 hr after injection. At this time, animals showed a decrease in activity and their fur had a rough appearance. Early deaths

occurred at 18 hr with the highest mortality rate occurring between 20 and 30 hr post-injection. Animals in the terminal stages of shock showed paralysis of the hind legs accompanied by diarrhea, and matted crusted eyes. Post-mortem examination of these animals failed to indicate any major gross pathological changes. Microscopic examinations of tissues taken from all major organs were also evaluated. Small vascular hemorrhage with edema in the mesenteric capillary bed of the gut was the only histological change observed.

Because of the wide variation in the toxicity of the various extraction products, chemical studies were undertaken to determine whether these variations were due to differences in chemical composition.

*Chemical Studies.* The carbohydrate content of the various endotoxins was the first parameter evaluated (Table 2). Hexoses were determined on hydrolyzed endotoxin preparations by three separate colorimetric procedures. The amount of hexoses detected in the phenol water product ranged from 68 µg as determined by the anthrone method to 82 µg (cysteine-$H_2SO_4$). However, the value of 77 µg/mg as determined by the phenol-$H_2SO_4$ procedure was the most reproducible and probably represents a more accurate concentration of hexoses. In addition to being more reproducible, the phenol-$H_2SO_4$ gave the greatest degree of sensitivity as compared to the other two methods. The hexose content detected in the TCA endotoxin exhibited slightly lower values and had an average value of 63 µg/mg. On the other hand, the three remaining endotoxin preparations exhibited almost two-thirds less hexose content and these values were closely clustered together. They ranged from 22-28 µg by all three colorimetric procedures.

Further chemical studies failed to detect any free pentoses in unhydrolyzed preparations. However, the hydrolysis of endotoxin released components resembling pentoses, the quantities of which could be accounted for by release of ribose from RNA. The values for methylpentoses as determined by the cysteine-sulfuric acid method was 34 µg/mg in the material obtained by phenol extraction. This was approximately two-fold higher than those values obtained from the other four endotoxins.

The measured quantities of heptose, KDO, and hexosamine which are generally associated with the core region of the cell wall are also shown in Table 2. Preparations exhibiting the greatest toxicity contained the highest concentrations of these three sugars while less toxic preparations exhibited a correspondingly lower degree of sugar content. The heptose content of the phenol product was 30.4 µg/mg and 26 µg/mg in the TCA endotoxin. A value of 15.2 µg/mg was obtained upon analysis of the ethyl-ether preparation while a value of 13 µg/mg was found in the EDTA and hot water extraction products. 2-keto-3-deoxyoctanoic acid could be detected in products from all five extraction procedures; however, variation in content was again noted. The values obtained ranged from 13 µg/mg in the phenol product to 2.1 µg/mg obtained from EDTA prepared endotoxin. The hexosamine content of the endotoxin extracted by phenol and TCA was found to be 40 µg/mg, while the other three extraction products exhibited substantially lower values (Table 2).

Additional chemical evaluations of the extracted endotoxins are summarized in Table 3. All of the endotoxin preparations were found

to contain both DNA and RNA as measured by the diphenylamine and orcinol procedures. The highest content of DNA was found in phenol extracted material (270 µg/mg). The aqueous ethyl ether prepared endotoxin contained the next highest concentration (129 µg/mg) while TCA-extracted endotoxin was found to have 116 µg/mg. The endotoxins obtained by EDTA-lysozyme and hot water extraction had identical concentrations of DNA (39 µg/mg).

The amount of RNA found in the extracted lipopolysaccharides was also found to vary considerably between the five endotoxin preparations. A concentration of 190 µg/mg and 114 µg/mg was respectively found upon evaluation of phenol and EDTA-lysozyme prepared endotoxins. Similar values for RNA content were found in TCA prepared endotoxin (81 µg/mg) and hot water extracted preparations (88 µg/mg), while the ethyl-ether extraction product yielded a preparation containing the lowest content of RNA (40 µg/mg).

The protein content of the five products was estimated by a direct colorimetric procedure and by calculations from micronitrogen content. Protein estimation obtained by calculation were consistently higher than those obtained by the Lowry procedure. The ethyl-ether and EDTA-lysozyme preparations exhibited the highest content of protein was found to be 563 µg/mg and 516 µg/mg, respectively. However with the Lowry procedure, 357 µg/mg and 330 µg/mg were obtained. Lower protein content was also found in the three remaining preparations. With the exception of the TCA preparations none of the preparations showed good correlation between the protein values obtained by both methods. However, the protein values of the aqueous phenol preparations exhibited

a strikingly disproportionately low protein content of 54 µg/mg using the Lowry procedure while direct calculation from the micronitrogen determinations yielded 312 µg/mg. With this particular preparation as well as the others the micronitrogen method appears to be more reliable than the Lowry method.

In contrast to some of the other chemical constituents evaluated in extracted endotoxin preparations, the lipid content as determined by the alkaline hydroxylamine procedure showed no correlation with lethality. The concentration of lipid detected in the ethyl ether and EDTA-lysozyme products was 199 µg/mg and 159 µg/mg, respectively. This was higher than the more toxic phenol extract which had a value of 140 µg/mg. However, the highest lipid content was obtained with the TCA extracted material (234 µg/mg), while the lowest content, was found in hot water extracted preparations, 72 µg/mg. The phosphate content, both total and inorganic, was the last comparative parameter evaluated. The phenol-water and the TCA preparations had total phosphate contents which were three times higher than the remaining three products. Total phosphate values of 37 µg/mg in aqueous phenol extracts and 40 µg/mg in TCA extracts were obtained. Of the total phosphate, 10 µg/mg was inorganic in the phenol-water product while 17 µg/mg represented the inorganic fraction in TCA extracts. As seen in Table 3 the total phosphate values obtained from the remaining preparations were very similar. However, the values for the inorganic phosphate content were not similarly clustered.

## DISCUSSION

The data described herein clearly indicate that the method of cellular extraction plays a major role in obtaining toxic preparations as well as in their chemical composition. Similar observations have been found to hold true for members of the Enterobacteriaceae (16, 27). In addition, the strain of a given organism and its cultural conditions are additional variable factors which could conceivably affect the chemical and biological properties of endotoxin thus making it difficult to make meaningful comparisons with the data of other investigators. The results presented herein also indicate that the intraperitoneal route which is generally used in endotoxin studies by many investigators was not as effective as the intravenous route for demonstration of lethal activity in endotoxin preparations from *P. aeruginosa*. Consequently, on a dry weight basis the amount of endotoxin required to kill mice by the intravenous route was about half that required by the intraperitoneal route. However, this should not be unexpected since the rate of vascular absorption of endotoxin administered intraperitoneally as well as its persistence in the blood stream would be less effective than direct intravenous administration. Of particular interest was that administration of endotoxin intranasally proved to be ineffective in the concentrations tested. This was in contrast to the *in vivo* elastase studies previously reported from this laboratory in which the intranasal route was the most toxic of four routes (22).

None of our endotoxin studies are consistent with the report of

Homma *et al* who claim that both their aqueous phenol and Boivin preparations exhibited a mouse $LD_{50}$ value of 0.1 mg i.p. (19). It was felt that this toxicity value was comparable to those reported for members of the Enterobacteriaceae. However, our most lethal preparations (Westphal) are more closely correlated to those values reported for the related genus of *Xanthamonas* (36). Volk found that phenol extraction of these organisms yielded endotoxin which had $LD_{50}$ values which ranged between 500 to 750 µg when given i.p. to mice. Based on the general pathogenic properties of the members of the Pseudomonadales, the $LD_{50}$ values obtained for *Xanthamonas* and *P. aeruginosa* endotoxin are probably more realistic estimations of endotoxin potency than those reported by Homma *et al* (19).

The results presented herein are also in opposition to the findings of Michaels (25) who was unable to obtain endotoxin from three strains of *P. aeruginosa* using both the aqueous phenol or TCA procedures. Instead, it was found that our most toxic preparations were obtained by the aqueous phenol and TCA extraction procedures, both of which had $LD_{50}$ values quite close to one another. However, in earlier studies, Michaels and Eagon (24) reported an $LD_{50}$ value of 1.25 mg with *P. aeruginosa* lipopolysaccharide isolated by ethyl ether extraction when administered intraperitoneally. This value is lower than our $LD_{50}$ of 1.75 mg obtained in the present study, although again strict comparison of the results is difficult since their $LD_{50}$ value was determined after a seven day holding period. Furthermore, when these investigators treated lipopolysaccharide extracted by the ethyl ether method with either EDTA or lysozyme the toxicity of the

preparations was lost, although these results are in direct opposition to those of Homma *et al* who found no change in similarly treated phenol preparations (19). The findings of Michaels and Eagon (24) would be somewhat consistent with our results in that direct extraction of endotoxin from *P. aeruginosa* with EDTA and lysozyme yielded an endotoxin of very low lethality. The low degree of toxicity of such preparations most likely results from disruption of the structural integrity of the endotoxin molecules during the extraction procedure. As pointed out by Michaels and Eagon (24) the site of action of lysozyme and EDTA is probably lipid A. They suggested that lysozyme affects glycosidically linked D-glucosamine units present in the lipid A moiety. Whereas, the role of EDTA has been postulated by many investigators to remove divalent cations resulting in a loss of structural integrity of the lipopolysaccharide (13, 17, 20, 28). A recent report by Shands (34) lends support to the idea that toxicity and structure are closely aligned. Thus, when endotoxin from *Salmonella typhimurium* was treated with alkaline hydroxylamine, which cleaves ester bound fatty acids, the bilayer morphology of the lipopolysaccharide was changed. Concomitantly, this change resulted in loss of animal toxicity.

The general trend noted from the results on chemical composition of the extraction products is that the two most toxic preparations contained higher concentrations of those components generally associated with the core region of the lipopolysaccharide molecule. This was not only reflected in the hexose content, but was also found to apply to heptose, KDO, hexosamine and phosphate content. For example, the value for methylpentose obtained with material extracted by the aqueous phenol method

was twice the amount as that found in the other endotoxin preparations. This higher value may represent some alteration of the product by the extraction procedure which in turn affects the color reaction. Similar findings with *Serratia marcescens* endotoxin indicate correlation between carbohydrate content and toxicity, while none was found between toxicity and nitrogen, fatty acids, or hexosamine content (16). In addition, the aqueous phenol product from *P. aeruginosa* was found to repeatedly give abnormally low protein values by the Lowry procedure which did not correspond to protein content as calculated from micro-nitrogen content. Similar analytical difficulties were also encountered by Clarke *et al* (5) who noted a substantial difference in the protein content of aqueous phenol extracts of *P. aeruginosa* cell walls when analyzed by two independent methods.

Of particular interest is the apparent complexity of endotoxin from *P. aeruginosa*, especially since all five of our preparations contained nucleic acids in addition to the expected carbohydrates, lipids and protein. These findings seem to be compatible with the findings of others (5, 19, 26). For example, Fensom and Gray using phenol extracts were unable to free *P. aeruginosa* lipopolysaccharide from small amounts of nucleic acids (13). In addition, Homma *et al* (19, 26) were able to phenol-extract endotoxin from both intact cells as well as autolysates of *P. aeruginosa* which were composed to two components separable by zone electrophoresis. Component I consisted of a lipopolysaccharide-protein complex, while component II consisted of DNA, RNA, and a polyribose phosphate complex. However, lethality of the preparation was associated only with the component I fraction. On the other

hand, Rogers *et al* (31) found that EDTA extraction of *P. aeruginosa* liberates a protein-lipopolysaccharide complex which they felt was probably representative of the *in situ* form of native endotoxin. Other workers suggest that the lipopolysaccharide of *P. aeruginosa* cell walls resembles that of other gram negative organisms. Fensom and Gray (13) have concluded that the lipid moiety has a similar amino sugar backbone to that of lipids of enterobacterial lipopolysaccharides, but contain different hydroxy acids. Also, the very high phosphorus content of the polysaccharide moiety could not be accounted for by nucleic acid content. Much of the phosphorus has been shown to be acid labile and appears to be present in the form of ethanolamine pyrophosphate (9, 20). Presumably, the presence of these pyrophosphate groups may possess metal-binding properties in the cell wall and may thus help explain the bactericidal action of EDTA on *P. aeruginosa*.

TABLE 1. SUMMARY OF $LD_{50}$ VALUES OF ENDOTOXIN EXTRACTED FROM *Pseudomonas aeruginosa* BY VARIOUS PROCEDURES

| EXTRACTION PROCEDURE | MOUSE LETHALITY ($LD_{50}$) µg DRY WEIGHT | | |
|---|---|---|---|
| | INTRAPERITONEAL | INTRAVENOUS | INTRANASAL[a] |
| Phenol-water | 840 | 450 | - |
| Trichloroacetic acid | 947 | 589 | - |
| Ethyl-ether | 1755 | 1652 | - |
| EDTA-lysozyme | 4400 | 3552 | - |
| Hot water | 4000 | 6000 | - |

[a]No lethality was observed within the range of 1 µg to 1 mg of endotoxin/.05 ml. Solubility limitations prevented assay of concentrations above the latter value.

TABLE 2. CARBOHYDRATE CONTENT OF THE LIPOPOLYSACCHARIDE FROM *Pseudomonas aeruginosa* $C_9$

| CARBOHYDRATE AND PROCEDURE | Phenol-water | TCA | Ethyl-ether | EDTA-Lysozyme | Hot Water |
|---|---|---|---|---|---|
| | | | µg/mg dry weight | | |
| Hexoses Anthrone | 68.4 | 62.5 | 22.5 | 27.5 | 25.2 |
| Cysteine-$H_2SO_4$ | 82.0 | 60.4 | 29.5 | 28.4 | 25.6 |
| Phenol-$H_2SO_4$ | 77.14 | 63.01 | 39.5 | 27.5 | 24.24 |
| Free-Pentose | 0 | 0 | 0 | 0 | 0 |
| Methyl-Pentose | 34.4 | 15.3 | 12.74 | 15.3 | 14.02 |
| Heptose | 30.45 | 26.1 | 15.23 | 13.05 | 13.05 |
| 2-Keto-3-deoxy-octonoate | 12.96 | 7.2 | 4.68 | 2.16 | 3.24 |
| Hexosamines | 40.4 | 40.4 | 24.2 | 16.2 | 16.5 |

TABLE 3. ANALYSIS OF THE LIPOPOLYSACCHARIDE OF *Pseudomonas aeruginosa* $C_9$

| MATERIAL TESTED | Phenol-water | TCA | Ethyl-ether | EDTA-Lysozyme | Hot Water |
|---|---|---|---|---|---|
| | µg/mg dry weight | | | | |
| DNA | 270 | 116 | 129 | 39 | 39 |
| RNA | 190 | 81 | 40 | 114 | 88 |
| PROTEIN | | | | | |
| Lowry | 54 | 222 | 330 | 357 | 175 |
| Micronitrogen | 313 | 234 | 516 | 563 | 281 |
| LIPIDS | 140.0 | 234.0 | 199.1 | 159.2 | 72.2 |
| PHOSPHATE | | | | | |
| Total | 36.8 | 40 | 12 | 11 | 9.2 |
| Inorganic | 10 | 17 | 7.2 | 1.4 | 1.4 |

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